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(54) Title: ANTI-SENSE OLIGODEOXYNUCLEOTIDE TO FIBROGENIC CYTOKINES AND USE THEREOF			
(57) Abstract			
<p>The present invention relates to an anti-sense oligodeoxynucleotide to mRNA for fibrogenic cytokines which are secreted in large quantities in a wound site and then which stimulate the fibrinoid degeneration of the tissue. The anti-sense oligodeoxynucleotide according to the present invention is bound to the complementary sequence of mRNA for fibrogenic cytokines such as TGF-β, TNF-α, PDGF, etc., to inhibit the genetic expression thereof and thus can inhibit the scarring due to the production of fibrogenic cytokines and the fibrinoid degeneration at the wound site to treat the wound so as to have an appearance substantially identical to the normal tissue. Accordingly, the present invention also relates to the use of anti-sense oligodeoxynucleotide to fibrogenic cytokines as a scarring inhibitor.</p>			

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ANTI-SENSE OLIGODEOXYNUCLEOTIDE TO FIBROGENIC
CYTOKINES AND USE THEREOF

5 BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a novel anti-sense
10 oligodeoxynucleotide to fibrogenic cytokines, and more
particularly, to a novel anti-sense oligodeoxynucleotide
which inhibits the gene expression of fibrogenic cytokines
secreted in a wound site in a large amount to prevent scar
formation. The present invention also relates to the use of
15 anti-sense oligodeoxynucleotide to fibrogenic cytokines as a
scarring inhibitor.

Description of the Prior Art

20 Immunological response, inflammatory response and
tissue repair response play a function in protecting the
host organisms from the dangerous surrounding environment.
However, if such responses do not occur smoothly or occur
in excess, damage may be inflicted on the host organism.
25 One example of biological processes involving such responses
is the wound healing process which results in the formation
of a scar, as a tissue repair response to trauma, a surgical
operation, a burn and the like. The scar thus formed may
frequently be constricted so as to inhibit the growth of
30 tissue, lead to a functional disorder or deteriorate the
external appearance of the host.

The wound healing process is a very elaborate tissue
response in which acute and chronic inflammation, cell
35 migration, vascularization, accumulation of extra cellular
matrix (ECM), etc. successively occur. When a wound
occurs, blood vessels surrounding the wound tissue are
injured causing local bleeding and clotting at a wound site.

Fibrinogen present in the blood clot forms fibrin gel into which plasma proteins, such as fibronectin, are introduced. Further, other cells such as inflammatory cells, fibroblasts, vascularization cells, and the like, are introduced
5 into such gel to liquify the gel while fibronectin accumulates ECM components such as collagen, proteoglycan, etc. in the tissue surrounding the wound. As a result of this process, fibrin matrix, originally present in the tissue, is replaced with granulation tissue on which the scar is formed
10 over a period of time. ECM accumulation and, at the same time, keratinocyte migration result in the formation of an epithelial membrane which can prevent fluid loss and bacterial invasion. The procedures involved in this wound healing process can be accomplished by the interaction of immu-
15 nological cells, inflammatory cells and mesenchymal cells of the impaired tissue with various cytokines, such as transforming growth factor- β (TGF- β), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and fibroblast activating factor (FAF), and ECMs such as collagen, fibro-
20 nectin, tenascin and proteoglycan.

It has been found that among the various cytokines which can promote fibroblast proliferation and increase ECM accumulation in wound tissue, that especially TGF- β and PDGF
25 play the most important role. It has also been disclosed that TGF- β significantly increases fibroblast proliferation and further expression of the gene for ECMs such as collagen and fibronectin and that PDGF has little effect on collagen synthesis but greatly promotes the proliferation of the
30 fibroblasts.

In addition, according to a recent study it has been reported that in the case of fetal wounds the wound healing process does not result in scarring and causes a little
35 inflammatory response and cytokine secretion in comparison to those in adult wounds [see, Whitby, D.J. and M.W.J. Ferguson, 1991, Immunohistochemical localization of growth factors in fetal wound healing, Dev. Biol. 147: 207-215].

This reference, however, describes that upon the injection of TGF- β into the tissue surrounding a wound, scar formation may result even in the case of fetal wounds and further glomerulonephritis may be induced.

5

Since the relationship of scar formation with the presence of cytokines has been identified, it has been proposed that the control of cytokines may inhibit the formation of scar tissue. For instance, Shah M., et al. 10 (cited below) observed that on the basis of the fact that TGF- β is physiologically secreted in an excessive amount during the wound healing process, when a wound is artificially formed on a rat and then a neutralizing antibody (NA) for TGF- β is injected around the wound, the wound site 15 treated with the neutralizing antibody does not show any change in tensile strength of the tissue and heals to a more near normal skin tissue appearance, in comparison with the wound of the control group which is not treated with said antibody. See, Shah M., Foreman DM., Ferguson MWJ., 1992, 20 Control of Scarring in Adult Wounds by Neutralizing Antibody to Transforming Growth Factor, Lancet 339 : 213-214. In addition, in this experiment it has also been demonstrated that the wound treated with neutralizing antibody heals without any scarring; whereas, the wound site of the control 25 group, which is not treated with neutralizing antibody, shows more inflammatory cells and accumulates a larger amount of ECMs, such as collagen or fibronectin, in comparison with the wound treated with the neutralizing antibody. However, such neutralizing antibody has difficul- 30 ty in practical application because it is very expensive and is too unstable for storage.

Additionally, Border WA., et al. have demonstrated that as a result of the administration of decorin (which is one 35 of the proteoglycan ECMs and can be bound to the TGF- β receptor and therefore can act as an inhibitor to TGF- β) to a glomerulonephritis animal model, decorin can inhibit fibrinoid degeneration to prevent glomerulonephritis [see,

Border WA, Rouslahti E, 1992, Transforming growth factor- β in disease: The dark side of tissue repair, J. Clin. Invest. 90: 1-7]. However, protein agents such as decorin are difficult to permeate into cells because of their macro-
5 molecular structures, and therefore, can be administered only through the oral route. Accordingly, it is difficult to obtain an effective level of the drug at the desired wound site.

10 As the most traditional and general method, in addition to the above mentioned methods, the synthetic medicinal agents such as steroids have been practically applied. However, such medicinal agents have no direct inhibiting effects on the scar formation and further exhibit untoward
15 side effects.

DISCLOSURE OF INVENTION

On the basis of the above mentioned prior art, the
20 present inventor has sought to determine the means to prevent the formation of a scar at a wound site by inhibiting the production of fibrogenic cytokines themselves rather than inhibiting or blocking the action of fibrogenic cytokines as already produced. As a result, the present
25 inventor has determined that the use of a specific anti-sense oligodeoxynucleotide to mRNA for fibrogenic cytokines can provide the desired effect.

Therefore, it is an object of the present invention to
30 provide a novel anti-sense oligodeoxynucleotide to mRNA for fibrogenic cytokines which are secreted in large quantities at a wound site and which then stimulate the fibrinoid degeneration of the tissue.

35 It is a further object of this invention to provide a composition for scarring inhibition which comprises a sufficient amount of anti-sense oligodeoxynucleotide to fibrogenic cytokines to inhibit the genetic expression of fibrogenic

cytokines and thus inhibit scarring at the wound site.

It is a further object of this invention to provide a use of anti-sense oligodeoxynucleotide as a scarring inhibitor.

It is a further object of this invention to provide a method of producing the novel anti-sense oligodeoxynucleotide to fibrogenic cytokines in large quantities.

10

The foregoing has outlined some of the more pertinent objects of the present invention. These objects should be construed to be merely illustrative of some of the more pertinent features and applications of the invention. Many other beneficial results can be obtained by applying the disclosed invention in a different manner or modifying the invention within the scope of the disclosure. Accordingly, other objects and a more thorough understanding of the invention may be had by referring to the summary of the invention and the detailed description describing the preferred embodiment in addition to the scope of the invention defined by the claims taken in conjunction with the accompanying drawings.

25

BRIEF DESCRIPTION OF THE DRAWINGS

For a thorough understanding of the nature and objects of the invention, reference should be had to the following detailed description taken in connection with the accompanying drawings in which :

Fig. 1 is an electrophoretic diagram of anti-sense oligodeoxynucleotide to TGF- β 1 and 2 (lane 2 and 3), TNF- α (lane 4), PDGF 1 and 2 (lane 5 and 6) and ϕ X174/Hae III (molecular size marker; lane 1) ; and

Fig. 2 is a graph showing effects of anti-sense TNF- α on the secretion of nitrite mediated by γ IFN and LPS.

BEST MODE FOR CARRYING OUT THE INVENTION

In one aspect, the present invention relates to a novel anti-sense oligodeoxynucleotide to fibrogenic cytokines. More particularly, the present invention relates to a novel anti-sense oligodeoxynucleotide which is bound to a complementary sequence for mRNA of fibrogenic cytokines to inhibit the genetic expression of fibrogenic cytokine and thus substantially or completely prevents the scarring at the wound site of an animal.

When the anti-sense oligodeoxynucleotide to mRNA for fibro genic cytokines according to the present invention is applied to the peripheral tissue of a wound, it is bound to said complementary sequence for mRNA to form a hybrid which inhibits the genetic expression of fibrogenic cytokines, whereby the production of fibrogenic cytokines at the periphery of the wound is inhibited preventing the formation of scar.

20

The anti-sense oligodeoxynucleotide according to the present invention is anti-sense sequences to mRNAs for fibrogenic cytokines, for example, transforming growth factor- β (TGF- β), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1), interleukin-6 (IL-6) and particularly to mRNAs for TGF- β and PDGF which induce most strongly the proliferation of fibroblasts and the production of connective tissue.

30

Typical examples of anti-sense oligodeoxynucleotides according to the present invention are illustrated below.

- o Anti-sense oligodeoxynucleotide to mRNA for TGF- β includes:

35

5'-CCGAGAGCGCGAACAGGGC-3' (SEQ ID NO: 1)

5'-GGTGTGCTGGGGAGG-3' (SEQ ID NO: 2)

- 5'-GGCTGGGGGTCACCC-3' (SEQ ID NO: 3)
 5'-AGAGAGATCCGTCTC-3' (SEQ ID NO: 4)
 5'-CCCGGAGGGCGGCAT-3' (SEQ ID NO: 5)
 5'-GGCAAAAGGTAGGAG-3' (SEQ ID NO: 6)
 5'-GAAAGCTGAGGCTCC-3' (SEQ ID NO: 7)
 5'-GAGAAGGGCGCAGTG-3' (SEQ ID NO: 8)
 5'-GTGGAGGGGAGGCTT-3' (SEQ ID NO: 9)
 5'-TGTCTCAGTATCCCA-3' (SEQ ID NO: 10)

10 o Anti-sense oligodeoxynucleotide to mRNA for TNF- α includes:

- 5'-AGCTTTCAGTGCTCAT-3' (SEQ ID NO: 11)
 5'-GGTGTCTTTCCAGG-3' (SEQ ID NO: 12)
 5'-TAGCTGGTCTCTGC-3' (SEQ ID NO: 13)
 5'-CCTGCCTGGCAGCTT-3' (SEQ ID NO: 14)
 5'-GGTGGCGCCTGCCACGAT-3' (SEQ ID NO: 15)

o Anti-sense oligodeoxynucleotide to mRNA for IL-6 includes:

- 5'-TGTGGAGAAGGAGTTCAT-3' (SEQ ID NO: 16)

o Anti-sense oligodeoxynucleotide to mRNA for PDGF includes:

- 5'-CCAGCAGCGATTTCAT-3' (SEQ ID NO: 17)
 5'-AACGCGTAGATCGAG-3' (SEQ ID NO: 18)
 5'-ATCAGGCGCTCAGGC-3' (SEQ ID NO: 19)
 5'-GACCAGACGCAGGTA-3' (SEQ ID NO: 20)
 5'-ACAGGTGGACGCGGC-3' (SEQ ID NO: 21)

Although the anti-sense oligodeoxynucleotide according to the present invention can be prepared by conventional chemical nucleotide synthetic methods, for the ease of preparation it is preferably synthesized by means of a computerized automatic DNA synthesizer. During this synothetic procedure, the total base is protected with

noncytotoxic protecting groups to prevent the base from cleavage with nuclease. As the protecting group for this purpose, phosphorothioate group is most preferably used. However, since the change of the base sequence has substantially no influence on the pharmacological effect when anti-sense oligodeoxynucleotide is applied to the skin, the base may be synthesized and used in the unmodified, i.e. unprotected, form. However, since the unmodified sequence is very sensitive to endonuclease naturally occurring in tissue, the anti-sense oligodeoxynucleotide modified by phosphorothioate group which is relatively highly resistant to endonuclease is preferably used in order to lengthen the duration of the therapeutic effect at the desired tissue. Thus, it should be understood that the anti-sense oligodeoxynucleotide to fibrogenic cytokines according to the present invention includes both the unmodified form and the modified (i.e. protected) derivatives. Then, the synthesized anti-sense oligodeoxynucleotide is conjugated with linkers at both ends and is introduced into a suitable vector plasmid to construct the recombinant plasmid with which the host bacteria is transformed. The obtained transformant is cultivated under suitable conditions to express anti-sense oligodeoxynucleotide in a large amount, which is then digested with restriction enzymes to separate and obtain only the desired anti-sense oligodeoxynucleotide in a pure state.

Plasmids which can be preferably used in the above procedures include, for example, pSPT18, pSPT19, pBlue-script, pGEM-3, etc., with plasmid pBluescript being particularly preferable. In transforming with a recombinant plasmid comprising such plasmid and anti-sense oligodeoxynucleotide, the preferable host organism is E. coli HB101.

The anti-sense oligodeoxynucleotide thus prepared according to the above method can inhibit the production of fibrogenic cytokines, which are known to be secreted in large quantities at the periphery of the wound, as

mentioned above and therefore can be used as an inhibitor to scar formation. Thus, the present invention further relates to the use of a novel anti-sense oligodeoxynucleotide defined above as an inhibitor to scarring in warm-blooded mammals including human being.

When using the anti-sense oligodeoxynucleotide as the scarring inhibitor, anti-sense oligodeoxynucleotide to one kind of fibrogenic cytokines can be used alone or, if required, in an admixture with anti-sense oligodeoxynucleotides to one or more other fibrogenic cytokines. For example, the use of a mixture of anti-sense oligodeoxynucleotide to TGF- β and anti-sense oligodeoxynucleotide to TNF- α can provide more rapid wound healing effect.

The anti-sense oligodeoxynucleotide according to the present invention can be formulated into a pharmaceutically acceptable preparation, for example, injections, sprays, ointments, creams and the like, with pharmaceutically acceptable conventional carriers and then administered either systemically or locally on the wound lesion. The pharmaceutically acceptable conventional carriers which can be used for this purpose may include distilled water for injection, phosphate buffer, physiological saline, etc. in the case of injections; ethanol, propylene glycol, glycerin, propellant, etc. in the case of sprays; and polyethylene glycol, liquid paraffin, carnauba wax, etc. in the case of formulation for topical use such as ointments and creams.

Although the dose of anti-sense oligodeoxynucleotide to fibrogenic cytokines according to the present invention can be varied according to the extent, severity, etc., of the wound lesion and condition of the subject for application, the anti-sense oligodeoxynucleotide is generally used in an amount of approximately $10\mu\text{M}$ for each time, 3 to 5 times per a day and most preferably in an amount of $50\mu\text{M}$ per a day. Preferably, anti-sense oligodeoxynucleotide according to the present invention is topically administered to the

skin either in the form of creams, ointments, lotions and the like or by subcutaneous injection.

The anti-sense oligodeoxynucleotide according to the present invention acts only on the genetic expression procedure for fibrogenic cytokines involved in scar formation due to the fibrinoid degeneration of tissues and does not result in any untoward side effects or toxicity. Moreover, since it is used in an extremely low amount of about 10 μ M, the anti-sense oligodeoxynucleotide according to the present invention has substantially no side effects including drug allergy, light sensitivity reaction, etc.

The present invention will be more specifically illustrated by the following examples but it should be understood that the present invention is not limited to these examples in any manner.

EXAMPLE 1

20

Synthesis of anti-sense oligodeoxynucleotide 5'-CCGAGAGCGC-GAACAGGGC-3' to mRNA for TGF- β

GENE ASSEMBLER SPECIAL (Pharmacia, LKB), as an automized DNA synthesizer, which is equipped with bottles containing acetonitrile for washing cartridge which is an inert polymer on which the desired oligodeoxynucleotide is assembled by repetitive addition of nucleotides, dichloroacetic acid for removing base-protecting groups and monodisperse plastic beads for removing water, respectively, and is programmed so that base C(cytosine) is used as a starting material and the desired amounts of bases A(adenine), G(guanine) and C(cytosine) are introduced in the desired order, was operated at the room temperature for 2.5 hours to synthesize the desired anti-sense oligodeoxynucleotide 5'-CCGAGAGCGCGAACAGGGC-3' to TGF- β .

The linker sequences GGGCC which provide the cutting

site for Apa I were bound to both ends of the anti-sense oligodeoxynucleotide thus prepared above. The obtained nucleotide sequence was introduced into the Apa I-cutting site of plasmid pBluescript to obtain the recombinant expression plasmid. The recombinant plasmid thus obtained was introduced into the host bacterial strain E. coli HB101 to yield the transformant. The transformed E. coli HB101 was incubated in LB medium (Luria-Bertani broth 1L; Bacto-tryptone 10g, Bacto-yeast extract 5g, NaCl 5g, 1N NaOH 1ml) at 37°C for about 16 hours to express the desired anti-sense oligodeoxynucleotide. The isolated plasmid was digested with restriction enzyme Apa I to obtain the pure anti-sense oligodeoxynucleotide having the desired sequence. The absorbance of the resulting product was measured at 260nm using a spectrophotometer. The yield of the desired product was 92%. According to base sequence analysis and electrophoresis (Fig. 1) of the produced anti-sense oligodeoxynucleotide, it could be shown that the desired title anti-sense sequence was obtained by the above procedure.

EXAMPLE 2

Synthesis of anti-sense oligodeoxynucleotide 5'-AGCTTT-CAGTGCTCAT-3' to mRNA for TNF- α

GENE ASSEMBLER SPECIAL (Pharmacia, LKB), as an automated DNA synthesizer, which is equipped with bottle containing acetonitrile for washing cartridge, dichloroacetic acid for removing base-protecting groups and monodisperse plastic beads for removing water, respectively, and is programmed so that the desired amounts of bases A(adenine), G(guanine), C(cytosine) and T(thymine) are introduced in the desired order, was operated at the room temperature for 2.5 hours to synthesize the desired anti-sense oligodeoxynucleotide 5'-AGCTTTCAGTGCTCAT-3' to TNF- α .

The linker sequences AA which provide the cutting site

for Hind III were bound to both ends of anti-sense oligodeoxynucleotide thus prepared above. The obtained nucleotide sequence was introduced into the Hind III-cutting site of plasmid pBluescript to obtain the recombinant expression
5 plasmid. The recombinant plasmid was then introduced into the host bacterial strain E. coli HB101 to yield the transformant. The transformed E. coli HB101 was incubated in LB medium at 37°C for about 16 hours to express the desired anti-sense oligodeoxynucleotide. The isolated plasmid was
10 digested with restriction enzyme Hind III to obtain a pure anti-sense oligodeoxynucleotide having the desired sequence. The absorbance of the resulting product was measured at 260nm using a spectrophotometer. The yield of the desired product was 90%. According to base sequence
15 analysis and electrophoresis of the produced anti-sense oligodeoxynucleotide, it is shown that the desired antisense sequence was obtained by the above procedure.

EXAMPLE 3

20

Synthesis of anti-sense oligodeoxynucleotide 5'-ATCAGGCGCT-CAGGG-3' to mRNA for PDGF

GENE ASSEMBLER SPECIAL (Pharmacia, LKB), as an auto-
25 mized DNA synthesizer, which is equipped with bottles containing acetonitrile for washing cartridge, dichloroacetic acid for removing base-protecting groups and monodisperse plastic beads for removing water, respectively, and is programmed so that the desired amounts of bases A(adenine),
30 G(guanine), C(cytosine) and T(thymine) are introduced in the desired order, was operated at the room temperature for 2.5 hours to synthesize the desired anti-sense oligodeoxynucleotide 5'-ATCAGGCGCTCAGGG-3' to PDGF.

35 The linker sequences ATCG which provide the cutting site for Cla I were bound to both ends of the anti-sense oligodeoxynucleotide thus prepared above. The obtained nucleotide sequence was introduced into the Cla I-cutting

site of plasmid pBluescript to obtain the recombinant expression plasmid. The recombinant plasmid was then introduced into the host bacterial strain E. coli HB101 to yield the transformant. The transformed E. coli HB101 was incubated in LB medium at 37°C for about 16 hours to express the desired anti-sense oligodeoxynucleotide. The isolated plasmid was digested with restriction enzyme Cla I to obtain a pure anti-sense oligodeoxynucleotide having the desired sequence. The absorbance of the resulting product was measured at 260nm using a spectrophotometer. The yield of the desired product was 92%. According to base sequence analysis and electrophoresis of the produced anti-sense oligodeoxynucleotide, it could be shown that the desired anti-sense sequence was obtained by the above procedure.

EXAMPLE 4

Effect of anti-sense oligodeoxynucleotide to TGF- β on the genetic expression for TGF- β

A wound was experimentally induced in each animal of a group of animals to which anti-sense oligodeoxynucleotide to TGF- β as a typical fibrogenic cytokine was administered. Then, the effect of the administered anti-sense oligodeoxynucleotide on the expression of cytokine in the test group was determined in comparison with the control group to which no anti-sense oligodeoxynucleotide was administered.

1) After a Balb/C mouse was anesthetized with ether, hair on the back portion of mouse was completely removed by using a hair cutter and thioglycolic acid. The back portion from the center to the limbs was cut down on four positions at the same intervals up to just above the muscle which were not sutured to induce spontaneous healing. At that time, one group of mouse was topically treated with 50uM of anti-sense oligodeoxynucleotide (5'-CCGAGAGCGCGAA-CAGGGC-3') to TGF- β as prepared in Example 1, which was dissolved or suspended in vehicles suitable for topical

application to the skin, and the other group of mouse was used as the control group. In each group, the expression of fibrogenic cytokines was measured as the trauma healing period has passed.

5

Just after wound induction and 1, 2, 3, 5 and 10 weeks after the wound induction, the tissue of wound site was separated from each group of mice to collect the cells. The collected cells were washed with PBS which does not
10 contain Ca^{2+} , Mg^{2+} and RNase, and then were introduced into NP-40 lysis buffer [0.5% NP-40, 10mM Tris-Cl(pH8.0), 100mM NaCl, 3mM MgCl_2 , 1000 U/ml RNasin (Promega)] and centrifuged at 12000 rpm for 2 minutes to remove cellular nucleus. The supernatant can be then separated and used direct-
15 ly as an RNA source.

According to another method, 1×10^8 cells were dissolved in 4ml of GIT buffer [4M guanidine thiocyanate, 50mM Tris-Cl(pH8.0), 10mM EDTA(ethylenediaminetetraacetate), 0.5%
20 sodium lauryl sarcosine, 0.1M mercaptoethanol] and then loaded on 7ml of 5.7M CsCl buffer and centrifuged at 80000 rpm for about 2 hours at 25°C. Thus, the total RNAs which were attached to the bottom and wall of test tube were obtained. To separate poly(A)⁺ mRNA in a pure state, an
25 appropriate amount of oligotex-dT was added to the obtained RNAs with using TE buffer as the eluant. The mixture was heated for 5 minutes at 65°C, rapidly cooled with ice and then incubated with 1/10 volume of 5M NaCl for 5 to 10 minutes at 37°C. Thereafter, the culture solution was
30 centrifuged at 15000 rpm for 5 to 10 minutes. After removing the supernatant, the residue was mixed with sterilized distilled water, heated again at 65°C for 5 minutes and then centrifuged at 15000 rpm for 5 to 10 minutes. The resulting product was precipitated with ethanol and then dissolved in
35 diethylpyrrocarbonate(DEPC)-dH₂O to obtain the desired mRNA.

mRNA thus prepared above was denatured at 65 to 75°C

for 10 minutes. 1 to 2 μ g of the denatured mRNA, 0.5 μ g of oligo(dT)₁₂₋₁₈ (Pharmacia), 1 x Taq polymerase buffer (50mM Tris-Cl, pH8.3, 3mM MgCl₂, 250 μ g/ml BSA), 1.25mM dNTP, 2.5mM MgCl₂, 20 units of RNasin(BRL) and 100 units of 5 murine reverse transcriptase(BRL) were added to a RNase-free test tube to which DEPC-dH₂O was added to the final volume of 20 μ l. The whole mixture was thoroughly stirred and then reacted at 37°C for one hour [see, Molecular Cloning, Vol. II, 14, pp20-21]. Then, the mixture was 10 heated at 95°C for 5 minutes to terminate the reaction and treated with NaOH to obtain a single-stranded cDNA which is then used as a template for polymerase chain reaction(PCR).

1 x Taq polymerase buffer (50mM Tris-Cl, pH8.3, 3mM 15 MgCl₂, 250 μ g/ml BSA), 200 μ M of dNTP, 0.25 μ M (10pmol) of each of primers 5'-GGGAAATTGAGGGCTTTCGC-3' and 5'-CTGAAG-CAATAGTTGGTGTC-3', 2 μ g of cDNA as prepared above and 0.1 U of Taq polymerase were introduced into a 0.5ml Eppendorf test tube and then sterilized dH₂O was added to make the 20 reaction solution to the total volume of 10 μ l, which was then thoroughly mixed. Then the test tube was centrifuged for a short time and the reaction solution attached to the wall of the test tube was collected on the bottom of the test tube. This reaction solution was carefully 25 transferred to a glass-made capillary tube while keeping the tube to be free from foam and then both ends of capillary tube were melted and sealed under heating. Then, PCR amplification was carried out repeatedly 35 to 45 cycles using a thermal cycler (FTC-2000), with one cycle comprising 30 5 seconds at 95°C, 5 seconds at 55°C and 15 seconds at 72°C. The amplified DNA was subjected to an electrophoresis using 1% Seakem agarose gel, stained with 50 μ g/ml of ethidium bromide solution and then identified by using a UV transilluminator. As a result, it could be determined that in the 35 tissue of the mouse to which anti-sense oligodeoxynucleotide to TGF- β according to the present invention was administered the strength of ethidium bromide staining is much weaker than that in the tissue of the non-treated control group.

From this result, it could be shown that anti-sense oligodeoxynucleotide to TGF- β can be bound to the front part of a structural gene for TGF- β to specifically inhibit the expression of TGF- β depending on the dose used. Accordingly, on the basis of such a dose-dependent inhibition the amount of anti-sense oligodeoxynucleotide capable of completely inhibiting TGF- β expression can be determined according to the number of cells present in the wound site and then utilized as an ideal dose thereof which can induce the clinically effective treatment effect on the wound.

2) in situ Hybridization :

The desired TGF- β gene was inserted into the corresponding EcoRI-cutting site of plasmid vector pBluescript (commercially available from Clontech) to prepare the recombinant plasmid which is then introduced into E. coli HB101 to obtain a transformant. The transformed E. coli HB101 thus obtained is incubated to produce the desired plasmid in large quantities which is then purified with cesium chloride (CsCl) gradient centrifuge (1g/ml CsCl, 740 μ g/ml ethidium bromide + plasmid DNA, 100000rpm, 10hrs). Thereafter, in vitro transcription with SP6 (or T3) T7 RNA polymerase was carried out at 37°C for 1 to 2 hours using a RNA labelling kit (made by B.M. Co., Ltd.). After the transcription reaction is completed, the template DNA was cleaved with DNase I and precipitated with ethanol to recover the RNA probe as a residual product. The recovered RNA probe was dissolved in 50 μ l of 10mM DTT, 10mM Tris-HCl, 1mM EDTA (pH7.6) and then used in hybridization. Upon the reaction is completed, the same amount of neutralizing buffer (300mM CH₃COONa, pH6.0, 1% CH₃COOH, 10mM DTT) was added to the reaction mixture and then ethanol was added to precipitate the desired probe.

0.5 μ g of the probe as obtained above was mixed with 100 μ l of a hybridization reaction solution (consisting of 50% deionized formamide, 10mM Tris-HCl, pH7.6, 200 μ g/ml of

10 RNase-free tRNA, 1 x Denhardt solution, 10% dextran sulphate, 600mM NaCl, 0.25% SDS) and then 50 to 100ul of the mixture was loaded on each glass slide to which mouse wound tissue as prepared above 1) was attached, and was subjected to hybridization for 16 to 22 hours while covering the glass slide with a cover glass. After completion of the hybridization, the cover glass was removed from the glass slide in 5 x SSC at 50°C, and the hybridized product was treated with RNase to remove unnecessary signals.

10 For the hybridized product, the coloring reaction was induced by means of DIG ELISA DNA labelling and detection kit (B.M. Co., Ltd.). Specifically, the hybridized product was placed in 100mM Tris-HCl, pH7.5, 150mM NaCl (TS) for about 5 minutes, removed, placed again in a blocking solution wherein dry milk powder free from fat and sugar is dissolved in TS in the concentration of 1.5%, and then allowed to stand for 30 minutes at room temperature. Thereafter, 0.2 units of alkaline phosphatase- conjugated anti-digoxigenin antibody diluted in 500 to 1000 times volume of TS was added and the mixture was reacted for 30 to 60 minutes. After completion of the reaction, the product was washed twice with TS for 15 minutes each time and then briefly washed in 100mM Tris-HCl, pH9.5, 100mM NaCl, 500mM MgCl₂ (TSM). The product was then stained with nitroblue tetrazolium (NBT) diluted in TMS and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) solution for one to two days. Then, TE buffer (Tris-HCl 10mmol/L, EDTA 1mmol/L, pH8.0) was added to terminate the coloring reaction and the glass slide was mounted with aqueous crystal mount. The result was determined from the staining condition.

As a result thereof, in the control group not treated with anti-sense oligodeoxynucleotide mRNA for cytokines was slightly detected on the first day from wound development but could be detected mainly in macrophages on and after the third day. On the other hand, in the test group treated with anti-sense oligodeoxynucleotide the signal for

mRNA of cytokines was not detected. According to this, it can be clearly seen that the expression of cytokines can be inhibited by anti-sense oligodeoxynucleotide.

5 EXAMPLE 5

For the anti-sense oligodeoxynucleotide according to the present invention, the scarring inhibition activity was determined in the following manner.

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A wound having a diameter of about 6mm was artificially induced on the back of each rat of a group of 40 rats. The rats were then divided into two groups, each of which consisted of 20 rats. In the first group, the wound site
15 was treated by spraying 50 μ M of anti-sense oligodeoxynucleotide (5'-CCGAGAGCGCGAACAGGGC-3') to TGF- β as prepared according to EXAMPLE 1 (the anti-sense treated group). In the remaining one group, the wound was not treated with anti-sense oligodeoxynucleotide (the non-treated control
20 group). Thereafter, the collagen content and tensile strength of the wound site as an important parameter for scarring were determined at specific times after the wound was induced. From this result, the scarring inhibition activity of anti-sense oligodeoxynucleotide according to the
25 present invention could be shown. The results are represented in Tables 1 and 2.

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Table 1. Collagen content in wound portion ($\mu\text{g}/\text{mg}$ of tissue)

Time elapsed	Collagen content	Non-treated control group	Anti-sense treated group
1 day		125	70
3 days		159	82
7 days		208	96
14 days		261	254
50 days		405	350

Table 2. Tensile strength of wound portion(MPa)

Time elapsed	Tensile strength	Non-treated control group	Anti-sense treated group
1 day		-	-
3 days		0.12	0.20
7 days		0.65	0.87
14 days		0.87	0.94
50 days		11.53	13.06

As can be seen from the results shown in Tables 1 and 2, in the rats treated with anti-sense oligodeoxynucleotide to TGF- β according to the present invention the collagen content was significantly lower than that in the non-treated control group but the tensile strength was higher in spite of such low collagen content. In addition, the administration of anti-sense oligodeoxynucleotide according to the present invention did not cause a delay in wound healing and can treat the wound so that it appears

more like normal tissue in comparison with that in the control group. Thus, it can be seen that the anti-sense oligodeoxynucleotide according to the present invention provides a superior tissue regeneration property whereby the wound can be substantially perfectly treated to the extent that the wound site could not be visibly distinguished from the normal tissue.

Such results thus obtained can be assumed to be due to the effect of anti-sense oligodeoxynucleotide to TGF- β by inhibiting the expression of various cytokines such as TGF- β , occurring generally in the wound site and by inhibiting the division of fibrous cells and the overproduction of connective tissue caused by growth factor. In addition, the administration of such anti-sense oligodeoxynucleotide as soon as possible, preferably immediately after the wound occurs, increases its beneficial effect and can lower vascularization.

20 EXAMPLE 6

In vitro test using anti-sense oligodeoxynucleotide to TNF- α

The anti-sense oligodeoxynucleotide to TNF- α was injected into in vitro culture solution of mouse macrophage cell line RAW 264.7 to observe the inhibiting effect on the formation of nitric oxide (NO) which is known as being involved in anti-tumor and anti-neoplastic effects. This test is based on the fact that the formation of NO is caused by the expression of gene for nitric oxide synthetase (NOS) and NOS gene is closely related to the expression of TNF- α [see, Journal of Immunology 146, 114-120, 1991 and Journal of Immunology 149, 3290-3296, 1992].

200 μ l of mouse macrophage cell lines RAW 264.7 were placed into a 96-well plate (Nunc) and 200 μ l of DMEM medium (Dulbecco's Modified Eagle's Medium + 5% bovine fetal serum + 0.5% penicillin/streptomycin) containing 10U/ml of γ IFN

and 10ng/ml of LPS (lipopolysaccharide) were added thereto. In addition, anti-sense oligodeoxynucleotide to TNF- α was added in the concentration of 0.1 μ M, 1 μ M and 10 μ M, respectively. For the control, anti-sense oligodeoxynucleotide to TNF- α was not added. Thereafter, all the test tubes were incubated at 37°C for 48 hours. Then, according to the method of Ding, et al. [see, Journal of Immunology 141, 2407-2412, 1988], the secretion of nitrite mediated by γ IFN and LPS in the presence and absence of anti-sense oligodeoxynucleotide to TNF- α was determined on the basis of the coloring reaction of 100 μ l of culture solution with the same amount of Griess reagent to estimate the effect of anti-sense oligodeoxynucleotide on nitrite production. The result thereof is depicted in Figure 2.

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As can be seen from the results shown in Figure 2, the NO production is dose-dependently inhibited by anti-sense oligodeoxynucleotide. According to this result, it can be concluded that anti-sense oligodeoxynucleotide to fibrogenic cytokines including TNF- α can directly inhibit the expression of fibrogenic cytokines, such as TNF- α , TGF- β , etc., which have been known as being expressed when a wound occurs.

25

As noted above, since the anti-sense oligodeoxynucleotide to fibrogenic cytokines according to the present invention inhibits the genetic expression of cytokines such as TGF- β , TNF- α , PDGF, etc., which are secreted in large quantities in the wound site to stimulate scarring, it can be utilized as a medicinal agent useful for inhibiting scarring in wound site to treat the wound to the extent that the wound site cannot be visibly distinguished from normal tissue.

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The more pertinent important features of the present invention have been outlined above in order that the detailed description of the invention which follows will be better understood and that the present contribution to the

art can be fully appreciated. Those skilled in the art can appreciate that the conception and the specific embodiment disclosed herein may be readily utilized as a basis for modifying or designing other structures for carrying out the same purposes of the present invention. Further, those skilled in the art can realize that such equivalent constructions do not depart from the spirit and scope of the invention as set forth in the claims.

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WHAT IS CLAIMED IS :

1. An anti-sense oligodeoxynucleotide and derivatives thereof, characterized in that it can be bound to the
5 complementary sequence for mRNA of fibrogenic cytokine to inhibit the genetic expression of fibrogenic cytokine.

2. The anti-sense oligodeoxynucleotide as defined in claim 1, wherein the sequence of anti-sense oligodeoxy-
10 nucleotide is modified by a phosphorothioate group.

3. The anti-sense oligodeoxynucleotide as defined in claim 1, wherein the fibrogenic cytokine is selected from the group consisting of: transforming growth factor- β
15 (TGF- β), tumor necrosis factor- α (TNF- α), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epithelial growth factor (EGF), interleukin-1 (IL-1) and interleukin-6 (IL-6).

20 4. The anti-sense oligodeoxynucleotide as defined in claim 3, wherein the fibrogenic cytokine is selected from the group consisting of: TGF- β , TNF- α and PDGF.

25 5. The anti-sense oligodeoxynucleotide as defined in claim 4, wherein the fibrogenic cytokine is TGF- β and the anti-sense oligodeoxynucleotide has the following sequence to mRNA for TGF- β :

30 5'-CCGAGAGCGCGAACAGGGC-3' (SEQ ID NO: 1) ;
5'-GGTGTGGTGGGGAGG-3' (SEQ ID NO: 2) ;
5'-GGCTGGGGGTCACCC-3' (SEQ ID NO: 3) ;
5'-AGAGAGATCCGTCTC-3' (SEQ ID NO: 4) ;
5'-CCCGGAGGGCGGCAT-3' (SEQ ID NO: 5) ;
5'-GGCAAAGGTAGGAG-3' (SEQ ID NO: 6) ;
35 5'-GAAAGCTGAGGCTCC-3' (SEQ ID NO: 7) ;
5'-GAGAAGGGCGCAGTG-3' (SEQ ID NO: 8) ;
5'-GTGGAGGGGAGGCTT-3' (SEQ ID NO: 9) ; or
5'-TGTCTCAGTATCCCA-3' (SEQ ID NO: 10).

6. The anti-sense oligodeoxynucleotide as defined in claim 4, wherein the fibrogenic cytokine is TNF- α and the anti-sense oligodeoxynucleotide has the following sequence to mRNA for TNF- α :

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5'-AGCTTTCAGTGCTCAT-3' (SEQ ID NO: 11) ;
5'-GGTGTCTCTTCCAGG-3' (SEQ ID NO: 12) ;
5'-TAGCTGGTCTCTGCT-3' (SEQ ID NO: 13) ;
5'-CCTGCCTGGCAGCTT-3' (SEQ ID NO: 14) ; or
10 5'-GGTGGCGCCTGCCACGAT-3' (SEQ ID NO: 15).

7. The anti-sense oligodeoxynucleotide as defined in claim 4, wherein the fibrogenic cytokine is PDGF and the anti-sense oligo deoxynucleotide has the following sequence
15 to mRNA for PDGF :

5'-CCAGCAGCGATTTCAT-3' (SEQ ID NO: 16) ;
5'-AACGCGTAGATCGAG-3' (SEQ ID NO: 18) ;
5'-ATCAGGCGCTCAGGC-3' (SEQ ID NO: 19) ;
20 5'-GACCAGACGCAGGTA-3' (SEQ ID NO: 20) ; or
5'-ACAGGTGGACGCGGC-3' (SEQ ID NO: 21).

8. A pharmaceutical composition for inhibiting scar formation during healing of a wound at a wound site
25 comprising an effective amount of at least one of anti-sense oligodeoxynucleotide to mRNA for fibrogenic cytokine as defined in any one of claims 1 to 7.

9. The composition as defined in claim 8, including
30 anti-sense oligodeoxynucleotide 5'-CAACACGGGTTCAGGTAC-3' (SEQ ID NO: 25) to mRNA for TGF- β .

10. The composition as defined in claim 8, including anti-sense oligodeoxynucleotide 5'-CATGCTTTCAGTGCTCAT-3'
35 (SEQ ID NO: 26) to mRNA for TNF- α .

11. The composition as defined in claim 8, including anti-sense oligodeoxynucleotide 5'-CCAGCAGCGATTTCAT-3' (SEQ

ID NO: 17) to mRNA for PDGF.

12. The composition as defined in claim 8 further including a pharmaceutically acceptable carrier, adjuvant or
5 excipient.

13. The composition as defined in claim 8, formulated in the form one of the following preparations: an injection, a spray, an ointment or a cream.
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14. Use of anti-sense oligodeoxynucleotide to mRNA for fibrogenic cytokine as defined in any one of claims 1 to 7 as a scarring inhibitor.

15 15. A process for producing purely anti-sense oligodeoxynucleotide to mRNA for fibrogenic cytokine as defined in any one of claims 1 to 7 in large quantities, characterized in that :

anti-sense oligodeoxynucleotide to mRNA for
20 fibrogenic cytokine is synthesized by means of an automated DNA synthesizer;

a linker sequence is bound to each end of the synthesized sequence and then the combined sequence is inserted into a suitable plasmid to obtain a recombinant
25 plasmid;

a suitable host bacteria is transformed with the recombinant plasmid obtained above; and

the obtained transformant is incubated to express the desired anti-sense oligodeoxynucleotide in large
30 quantities.

FIG. 1

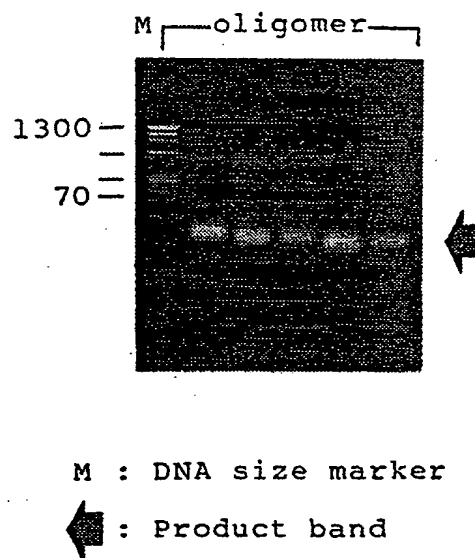
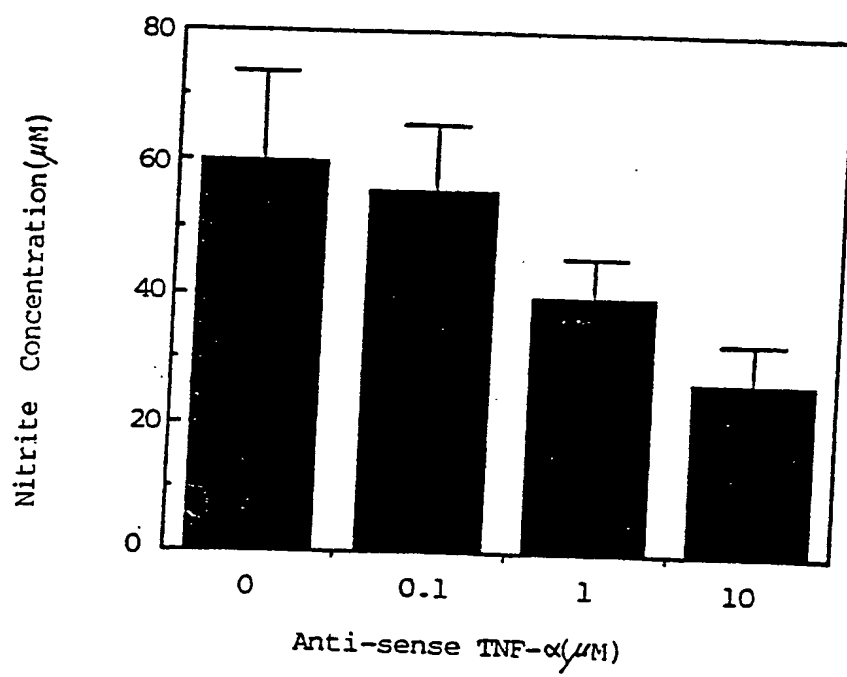


FIG. 2



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